MECHANICAL FORCES AS A CAUSE OF CELLULAR DAMAGE BY FREEZING AND THAWING

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During microscopic examination of mammalian red blood cells exposed to freezing and thawing Smith, Polge and Smiles (1951) did not observe any intracellular ice crystals between the time the ice formed in the surrounding Ringer's solution and the time the cells became hemolyzed. When amoebae in pond water were subjected to a similar procedure, intracellular ice formation occurred. The presence of internal ice crystals in the amoebae was always associated with rupture of cell membranes and the amoebae in question never revived. Their experiments reveal that there may be differences among animal cells in their responses to freezing at a fixed temperature. The role played by ice formation *per se* is uncertain.

There are several potential factors involved with freezing injury to animal cells. On the one side we have the mechanical forces attendant upon ice crystal formation and on the other the physical and chemical changes such as hypertonicity or shift in pH associated with withdrawal of water from solution. The mechanical factors involved must be expected to be less dependent on the length of time the cells are exposed to freezing than are the physical and chemical ones. Intracellular ice formation is most likely to occur in supercooled cells (Mazur, 1963). Cells will become supercooled at freezing rates which are so high that intracellular water can not pass through the cell membrane rapidly enough to keep the concentration of solutes inside the cell in equilibrium with that of its surroundings. Assuming similar qualities for their cell membranes a suspension of small cells would therefore have to be exposed to more rapid freezing to show intracellular ice formation than would a suspension of large cells. The red blood cells of the congo eel have a diameter which is 10 times the diameter of the mammalian red blood cells studied by Smith, Polge and Smiles (1951). Visual observation during freezing at the freezing rate required to produce intracellular crystallization in mammalian red blood cells may be impossible, while the red blood cells of the congo eel are likely to become frozen internally at freezing velocities which permit observations of the freezing process. For this reason red blood cells from congo eels were utilized in an attempt to disclose whether freezing injury due to mechanical forces could be observed separate from eventual damage resulting from chemical changes. The cells could be studied during freezing and thawing over periods short enough to avoid hemolysis produced by changes other than the mechanical ones associated with ice crystal formation.

Methods

Fresh samples of venous blood from the congo eel (*Amphiuma tridactylum*) with nucleated red blood cells of a diameter in the range of 70–90 μ were diluted

with Ringer's solution and a drop spread on a 1-mm.-thick slide of the acrylic resin "Perspex" and covered with a 0.2-mm. coverslip. A small piece of solid carbon dioxide in tinfoil was then placed at the edge of the coverglass, and the freezing of the diluted blood was watched through a Leitz Ortholux microscope with Leica camera for photomicrography. Magnifications between 40 × and 200 × were used. A thin layer of glycerol on the coverglass facilitated observation by avoiding condensation of water.

Results

With the piece of solid carbon dioxide in place, ice formation in the diluted blood began immediately. The ice crystal front grew into the preparation and

Figure 1. The ice front is advancing through diluted blood from the congo eel. Shrinkage of the red blood cells starts as soon as they are reached by the spear-shaped ice crystals. The volume occupied by ice compared to the volume of the fluid space among the ice crystals indicates the portion of water withdrawn from solution. Magnification 100 ×.

water was withdrawn from solution. Shrinkage of the red blood cells started when they were reached by the ice front (Fig. 1) and continued concurrent with the decrease of fluid space among the ice crystals. At first ice crystals appeared exclusively in the suspending medium. Behind the ice front, however, ice crystals formed suddenly within one after the other of the shrunken cells. The crystals were so small that the light was scattered and the cell interior became opaque and appeared quite black (Fig. 2, A). When a definite extent of extracellular ice formation and thereby a certain degree of cooling was reached, the phenomenon would occur at various distances behind the ice front. The spontaneous intracellular freezing took place only as long as the ice front was advancing. The opaque

area sometimes appeared to occupy the whole cell interior, but in most experiments only the nuclear area and its immediate surroundings became opaque. If the piece of solid carbon dioxide at the edge of the coverglass was removed when a few of the red blood cells were frozen internally, the ice front receded (Fig. 2, B). With the exception of the cells with intracellular ice crystals the shrunken cells regained their shape, but some of them showed a ragged cell surface (Fig. 2, C). When

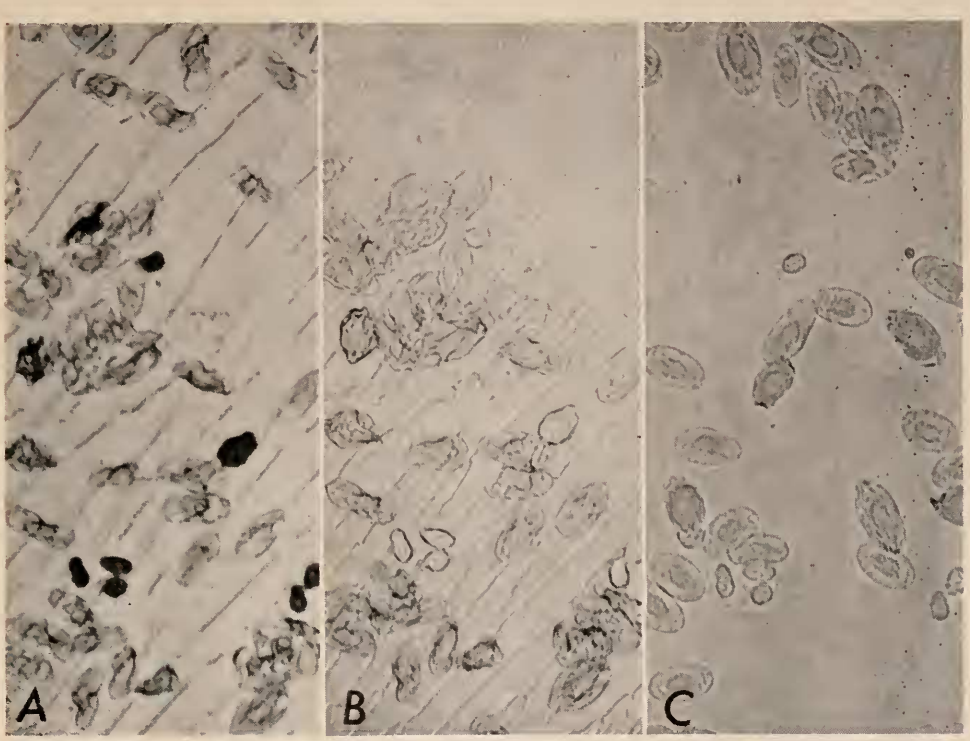

FIGURE 2. Diluted blood from the congo eel observed in three successive situations, A, B, and C, all observations made on the same field. A: The whole field is frozen and in a few of the cells intracellular ice formation is visibly manifest from the black appearance of their nuclear area. B: The ice front is receding. Dehydrated cells without intracellular ice crystals start regaining their normal shape. The opacity of the nuclear area of cells with intracellular ice crystals disappears. C: Thawing is complete. Some cells which have been dehydrated show a ragged cell surface, but only the cells with intracellular ice crystal formation are hemolyzed. The nuclei from the hemolyzed cells are left intact. Due to movements of fluid during thawing many of the cells have changed position, but five cells with intracellular ice crystals can be observed (as groups of three and two cells, respectively) in the lower part of the field (A). These cells are easy to trace through B and C. Magnification 100 ×.

the ice crystals of the internally frozen cells melted, the cells did not imbibe water to return to normal size. Their hemoglobin simply spread out into the suspending medium and apparently intact nuclei were left at the former sites of the whole cells (Fig. 2, C).

The ice front advanced more rapidly the nearer to the piece of solid carbon dioxide the field under observation was chosen. This was reflected in the sequence

of events as observed through the microscope. The description above and both figures refer to a field some distance away from the piece of solid carbon dioxide. Here the individual cells were easy to follow both during freezing and thawing. Further away the ice front advanced very slowly, and the cells seemed to become completely dehydrated without any signs of intracellular ice formation. Close to the edge where the freezing was initiated intracellular ice formation occurred almost immediately in all cells and dehydration could hardly be observed. Estimation of freezing temperatures from the amount of fluid space between extracellular ice crystals indicated that the slowly frozen cells would tolerate short-time freezing to lower temperature than the temperature at which all cells were destroyed at high freezing velocities.

By adjusting the size of the piece of solid carbon dioxide a frozen area which never reached the opposite edge of the coverglass was obtained. Different fields within this frozen area were kept under observation for 2–3 minutes with the ice front stagnant. The cells nearest to the edge where freezing started were all frozen internally. Somewhat further away some cells were frozen internally and some were dehydrated but unfrozen. Towards the ice front only dehydrated cells were found. Upon thawing all cells in the previously frozen area were hemolyzed except for a few ones close to the ice front.

Discussion

The observations made during the present investigation show that two potential factors in cellular injury from freezing and thawing can be studied as separate processes by choosing appropriate experimental material and freezing rates. As an advocate for the great role of physical and chemical factors in freezing injury to animal cells, Lovelock (1953, 1957) presented experimental support for a theory involving a mechanism for cellular damage by freezing which would act without intracellular formation of ice. He showed that phospholipids and cholesterol were lost from the membranes of red blood cells suspended in solutions of sodium chloride, and that this loss was augmented with increasing concentration. The results were identical whether this increased concentration was brought about by freezing or by the addition of sodium chloride to the initial solution. The red blood cells in this way became more permeable to cations, and with an excess of cations the cells would slowly swell and hemolyze. Eventually they would be rapidly entered by water molecules and immediately hemolyzed when returned to physiological saline during thawing. The slowly frozen red blood cells from the congo eel which were dehydrated without any signs of intracellular ice formation and yet became hemolyzed if kept in this state for a few minutes before thawing apparently were destroyed according to this theory.

Sloviter (1962) emphasizes the possible damaging effect of mechanical forces attendant upon the formation of ice. He found that the extent of hemolysis after freezing and thawing of mammalian red blood cells suspended in non-electrolytes in the presence of different concentrations of sodium chloride did not increase with an increase in ionic strength of the surrounding medium of the cells. The destruction of red blood cells by sudden intracellular ice formation, as shown in Figure 2 in the present paper, demonstrates that such a mechanism for cellular

injury may occur. Although this process as studied in the present investigation seemed to be closely associated with the formation of ice crystals inside the cells, a weakening of the cell membranes, due to increased concentration of salts, may of course precede and promote the intracellular freezing. External ice crystals may also tear or penetrate the cell membrane and induce ice formation in supercooled cells.

The ice formed inside the red blood cells of the congo eel was not observed as individual ice crystals, but as a result of a scattering of the light when the crystals occurred in great number. The opaque area in most experiments covered only a field somewhat greater than the cell's nucleus, corresponding to the thickest part of the cell. Supercooling followed by rapid intracellular crystallization would be expected to promote the formation of ice throughout the cell. The failure to observe individual ice crystals in the thin portions of the cell may perhaps be ascribed to the low magnification used in this study. That supercooling is necessary for intracellular ice formation is indicated by the fact that the process was always associated with an advancing ice front. When the ice front is stagnant the individual cells are exposed to a constant temperature and apart from an eventual recrystallization with changes in ice crystal size, no alterations in the suspending medium will take place. The concentrations of solutes inside the cells will be in equilibrium with those of the outside. Very slow freezing will also allow equilibrium to be maintained and supercooling will be minimal.

The process of sudden intracellular ice formation with the appearance of cell opacity during freezing was described by Smith, Polge and Smiles (1951) to occur at about −8° C. for the amoeba. After thawing of the amoeba, its cell membrane was ruptured and cytoplasmic granules drifted out into the medium. Smith and Smiles (1953) found the same phenomenon to take place between −6° and −12° C. in preparations of tissues from guinea pig testis. The internally frozen cells from guinea pig testis were also disintegrated after thawing. This shows that the response of the red blood cells of the congo eel to freezing and thawing is related to the response of other cell types investigated, but intracellular ice crystal formation seems not to be inevitably lethal. Salt (1959) has demonstrated that the large cells in the fat body of the goldenrod gall fly, *Eurosta solidaginis,* can survive freezing even if intracellular ice crystals have been present.

Considering the frozen area where the ice front was kept stagnant for some minutes as described in the present paper, it appears that several ways of damage are demonstrated. Near the piece of solid carbon dioxide rapid freezing with intracellular ice formation, which destroyed all cells, occurred. A short distance behind the ice front the exposure to increased salt concentration during freezing probably was the only cause of injury. At an intermediate distance some cells seemed to be destroyed by one of these processes and some by the other one. The few cells quite close to the ice front which were found to recover after thawing show that freezing at low velocity to temperatures just below zero is not fatal provided the exposure is of short duration. This may correspond to the finding of Lovelock (1953) that the critical temperature range for red blood cells starts at −3° C. Once ice is formed in the preparation, the temperature at the ice front will be equal to the freezing point of the solution, and a temperature of −3° C. or lower must therefore be sought a short distance behind the ice front and

towards the piece of solid carbon dioxide. Judged from the present investigation it appears that, dependent on cell type and cooling rate, mechanical forces may be the predominant factor in cellular damage from freezing and thawing under some circumstances as may physical and chemical changes under others.

To avoid intracellular ice formation it is necessary to cool the cells at a slow rate. That rapid cooling promotes intracellular freezing is also shown for sea urchin eggs (Asahina, 1961) and for yeast cells (Nei, 1960; Mazur, 1961). However, during slow cooling the time of exposure of the cells to increased salt concentrations is prolonged as long as the temperature is above the eutectic points of these solutes. To obtain minimum damage two requirements therefore are to be met with. The cells must be cooled slowly enough to prevent intracellular ice formation and rapidly enough to minimize the damaging effects of exposure to increased salt concentrations. It is interesting, then, to note that for mammalian red blood cells such an optimum cooling velocity for minimum hemolysis has been demonstrated (Gehenio and Luyet, 1958).

I am grateful to Dr. Joseph Engelberg for careful reading of the manuscript. The investigation was supported by a research training award from the Norwegian Research Council for Science and the Humanities.

Summary

1. The present investigation was initiated in an attempt to dissociate two potential factors in cellular injury from freezing and thawing: damage due to mechanical forces attendant upon ice crystal formation and damage due to physical and chemical changes associated with withdrawal of water.

2. Red blood cells in diluted blood from the congo eel were covered and subjected to microscopic examination during freezing induced by a piece of solid carbon dioxide placed at the edge of the coverglass. Ice crystals grew into the preparation, first rapidly and then more slowly the further from the piece of solid carbon dioxide they advanced. The sequence of events as observed through the microscope was different for different freezing velocities.

3. Rapid freezing caused intracellular ice formation and this internal freezing was always associated with hemolysis even if followed by immediate thawing. At slow freezing the cells became dehydrated without any signs of intracellular ice formation. Such cells would recover if thawing occurred within a few seconds, but they were all hemolyzed after prolonged exposure.

4. It is concluded that, dependent on the freezing rate, either mechanical forces or physical and chemical factors may be the main cause of cellular damage from freezing, the mechanical forces being predominant at rapid freezing.

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